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Favorable Outcome in Patients with Acute Myelogenous Leukemia with the Nucleophosmin Gene Mutation Autografted after Conditioning with High-Dose Continuous Infusion of Idarubicin and Busulfan

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Mutations of the nucleophosmin gene (NPM1), in the absence of concurrent FLT3-internal tandem duplication (FLT3-ITD) have impressive prognostic value in patients with acute myelogenous leukemia (AML), carrying normal karyotype (NK). In this study we describe treatment results from a series of 19 patients with NPM+/FLT3− autografted in first complete remission (CR) after conditioning with a regimen, named BuI, based on high-dose continuous infusion of idarubicin and Busulfan. Ninety-nine consecutive patients (median age of 54 years) with NK AML autografted in first CR were analyzed. Nineteen of 99 patients (19%) had NPM1 mutation in the absence of FLT3 mutations. The control group, accounting for 80 patients, included 16 cases (15%) with both mutations, 10 (12%) with FLT3/ITD mutation and no NPM mutation, and 54 (68%) in whom neither NPM1 nor FLT3 mutations were detectable. The median overall survival (OS) for the whole patient population was 34 months, the median disease-free survival (DFS) was 22 months. Median OS and DFS were significantly longer for patients with isolated NPM1 mutation as opposed to controls (OS: not reached versus 25 months, $P = .02$; DFS: not reached versus 16 months, $P = .007$, respectively). Of interest, patients with isolated NPM1 mutation had a better outcome in terms of either OS or DFS compared to the group of 16 NMP1+/FLT3+ patients. In conclusion, our study suggest that BuI regimen results in favorable clinical outcome in patients with isolated NPM1 mutation, and could be investigated in a randomized study versus other regimes or repeated courses of high dose cytosine-arabinoside.

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INTRODUCTION

The clinical outcome of acute myelogenous leukemia (AML) is extremely variable, ranging from survival of a few days to cure [1,2]. Different clinical and biological features at diagnosis have been reported as useful for the prediction of clinical outcome [3]; however, in most AML cases induction therapy must be initiated as soon as possible; therefore, the possibility of stratifying patients at diagnosis is generally not taken into account, with the exception of acute promyelocytic leukemia, in which morphology, immunophenotype, and molecular biology allow a rapid diagnosis and the adoption of specific therapy [4]. As a consequence, prognostic factors in AML are more useful for the prediction of relapse, rather than for the stratification of induction therapy [1]. Most relevant studies, based on large multicenter trials, have definitively demonstrated that age and cytogenetic abnormalities at diagnosis are the most important prognostic determinants for patients with AML [5-7]. However, nearly half of AML patients present with normal karyotype (NK) and are classified as intermediate in the prognostic categorization [6]. In recent years, new molecular markers have emerged as significant prognostic parameters; in particular, in patients with NK, mutations of the nucleophosmin gene (NPM1), in the absence of concurrent FLT3-internal tandem duplication (FLT3-ITD) have impressive prognostic and, beyond prognostication, predictive

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properties [8-11]. This NPM1+/FLT3-ITD− genotype predicts equivalent favorable outcome after intensive chemotherapy (CHT) and allogeneic stem cell transplantation (allo-SCT), whereas in the absence of this marker clinical outcome was significantly improved after an allogeneic transplantation [12]. More recently, the favorable impact of NPM1 mutation has also been reported for AML patients in advanced age, independently by transplantation procedures in the postremission phase [13]. Although there is a general consent that allo-SCT have no role in patients with NPM1 mutated AML in first complete remission (CR), no study has specifically focused on the therapeutic results of autologous SCT (ASCT) in NK AML patients presenting with NPM1+/FLT3− genotype [14]. However, the better responsiveness to CHT of NPM1+ patients seems to be, at present, a common finding observed in evidence by all clinical trials; accordingly, NPM1+ patients could be ideal candidate to ASCT, given that the ASCT does exert its antileukemic efficacy by targeting leukemic residual cells after induction/consolidation CHT by conditioning regimen, in turn, based on high-dose CHT [15]. Either in older or in young adult patients with AML, we previously reported encouraging results in terms of disease-free survival (DFS) and toxicity of an original conditioning regimen, called BuI, based on the combination of high-dose continuous infusions (c.i.) of idarubicin (IDA) and oral busulphan (Bu). The uniqueness of the regimen used in our series lies with the specific use of high dose IDA, in combination with classical dose of oral Bu and the removal of cyclophoshamide (Cy), which is not included in induction, consolidation, or salvage treatment for AML and, therefore, would exert minimal effect on leukemic population [16,17]. More recently, we replaced oral with intravenous Bu, achieving substantial reduction of nonhematologic toxicity, namely, oral mucositis [18]. In this study we describe the clinical characteristics and treatment results from a series of 19 patients with NPM+/FLT3− autografted in first CR after conditioning with high-dose continuous infusion IDA and oral or intravenous Bu.

PATIENTS AND METHODS

Ninety-nine consecutive patients with normal karyotype AML, treated at our Institutions between April 2003 and June 2009, in which ASCT was actually given in first CR, were analyzed. There were 62 male patients (64%) and 37 female patients (36%), with a median age of 54 years (range: 15-77). Cytogenetic RHG-banding analysis was performed with a standard method and the definition of a cytogenetic clone and descriptions of karyotypes followed the International System for Human Cytogenetic Nomenclature [19]. A minimum of 20 bone marrow (BM) metaphases/case were required to be examined for a case to be classified as having NK. Diagnosis was made according to WHO classification, by using >20% BM blasts as threshold for the diagnosis of AML [20]. In all cases, diagnosis was confirmed by immunophenotypic study, as previously described [21]. Overall 83 patients (84%) were diagnosed as having de novo AML, whereas in 16 patients (16%) AML arose after a previously diagnosed myelodysplastic syndrome (MDS).

The analysis of FLT3 mutation was performed as previously described [22]. Analysis of NPM1 mutations in AML cells was performed by fragment analysis as previously published [23]. In detail, genomic DNA was isolated from mononuclear cell preparations stored at −70°C using the DNAzol reagent (GibcoBRL, Eggenstein, Germany) according to the manufacturer's recommendations. Polymerase chain reaction (PCR) amplification of NPM1 exon 12 was carried out using primers NPM1-F (5′-TTAACTCTCTGGTGGTA GAATGAA-3′) and NPM1-R (5′-CAAGACTATT TGCCATTCCTAAC-3′), as follow. The total reaction volume of 50 μL contained approximately 100 ng DNA, 10 pmol of each primer, deoxynucleoside triphosphates (dNTPs, 10 mM each), 2.5 U Gold Taq polymerase, and supplied buffer (Applied Biosystems, Darmstadt, Germany). Samples were amplified using the following PCR conditions: 95°C for 5 minutes; 40 cycles of 94°C for 30 seconds; 55°C for 1 minute; 72°C for 1 minute. PCR products were screened for the presence of point mutation by Denaturing high-performance liquid chromatography (D-HPLC). PCR products were purified by standard methods and directly sequenced with primer NPM1-R2 (5′-GGCATTTT-GGACAACACA-3′) using the ABI Ready Reaction Dye Terminator Cycle Sequencing Kit (Applied Biosystems).

Patients aged up to 60 years with de novo AML were given induction treatment consisting of ICE (idarubicin 10 mg/sqm on days 1, 3, and 5; cytarabine [ARA-C] 100 mg/sqm as c.i. on days 1-7; etoposide 100 mg/sqm on days 1-4). Following achievement of CR, patients were consolidated with the NOVIA regimen (ARA-C 500 mg/sqm every 12 hours on days 1-6 and mitoxantrone 10 mg/sqm on days 4-6). Granulocyte colony-stimulating factor (G-CSF) (10 mg/kg) was added on day 15 from the start of NOVIA to induce mobilization of CD34-positive ($CD34^+$) cells. In patients failing to mobilize after NOVIA (n = 5, 2 NPM1+ and 3 controls), a further attempt was made with 1 cycle of high-dose ARA-C (3 g/sqm every 12 hours on days 1, 3, and 5). In patients aged over 60 years, induction treatment consisted of continuous sequential infusion of fludarabine (Flu) and ARA-C as previously reported [24]. Briefly, Flu was administered at a loading dose of 10 mg/sqm over 15 minutes at day 0 followed by a c.i. of 20 mg/sqm per 24 hours for 72 hours; ARA-C was given at a loading dose of 390 mg/sqm (infusion

Table 1. Characteristics of Patients

	NPM1+	Controls	*P*-Value
Patients' number	19	80*	
Age, median, (range)	55 (21-72)	52 (15-77)	.26
≤60 years/>60 years	10/9 (53%/47%)	64/16 (80%/20%)	.07
Sex (M/F)	9/10 (47%/53%)	53/27 (66%/34%)	.10
de novo/secondary	18/1 (95%/5%)	65/15 (81%/19%)	.13
WBC at diagnosis ($\times 10^9$/L)	14.8 (2.4-112)	7.3 (1.2-56.4)	.003
% BM blasts	80 (30-100)	55 (24-99)	.03
% PB blasts	50 (2-90)	75 (5-100)	.04
Serum LDH (IU/L)	875 (440-1830)	639 (340-1120)	.01
Total numberof $CD34^+$ cells collected ($\times 10^6$/kg)	6.5 (3.4-45)	6.8 (2.8-60.1)	.12
Oral Bu/i.v.Bu	13/6 (68%/42%)	62/18 (77%/23%)	.35

BM indicates bone marrow; PB, peripheral blood; WBC, white blood cell; Bu, Busulfan; LDH, lactate dehydrogenase; AML, acute myelogenous leukemia.
*Sixteen patients had NMP1+/FLT3+ AML; 64 patients had neither NPM1 nor FLT3 mutations.

duration: 3 hours) 3.5 hours after Flu and then as c.i. at 1440 mg/sqm per 24 hours for a total of 96 hours. G-CSF was added on day 15 at a dose of 5 mg/kg. Following CR, patients were given an additional course reduced by 1 day (c.i. Flu for a total of 48 hours and c.i. ARA-C for a total of 72 hours) and G-CSF at 10 mg/kg was added at day 15 to shorten neutropenia and mobilize hematopoietic precursors. The conditioning regimen, called I-Bu, consisted of 3 days of c.i. idarubicin (20 mg/sqm from days −13 to −11) followed by oral Bu (4 mg/kg daily) for 4 days (from days −5 to −2). Patients aged over 60 years received the same conditioning regimen reduced by 1 day for either idarubicin or Bu [17]. In 24 patients, oral Bu was replaced with intravenous Bu at a slightly reduced dose (3.2 mg/kg daily from days −5 to −2) as recently published [18]. To prevent seizures, phenytoin (100 mg) was administered orally beginning 24 h before the first dose of Bu and continued until 24 h after the last dose. All transfused blood products were depleted of leucocytes to minimize the risks of alloimmunization and graft-versus-host disease (GVHD). Infection prophylaxis was based on oral ciprofloxacin when neutrophils were $<1 \times 10^9$/L. Neither antiviral nor antifungal prophylaxis was performed. Indications for antibiotic therapy included fever >38°C with leukocyte count $<1 \times 10^9$/L as well as signs or symptoms of infection. Intravenous fluconazole was used for proven candidiasis infection, whereas amphotericin B was given for aspergillosis when suspected (fever persisting for more than 7 days while on treatment with broad-spectrum antibiotics) or proven. G-CSF (5 mg/kg) was added for all patients at day +5 from stem cell infusion. The median number of $CD34^+$ cells collected was 6.7×10^6/kg (range: 2.8-60.3). The median number of $CD34^+$ cells infused was 6.2×10^6/kg (range 2.8-24). DFS was defined as the time from CR achievement until the month of relapse, death from any cause, or the date of last follow-up. Overall survival (OS) was defined as the time from diagnosis to death or last follow-up. Either DFS or OS were calculated by the Kaplan-Meier method [25]. Differences between survival curves were evaluated by the log-rank test. Differences in the distribution of individual parameters among patient subsets were analyzsed using the chi-square or the Mann-Withney test. All statistical comparisons used 2-tailed *P*-value. Multivariate analysis was performed by a Cox proportional hazard regression model. The *P*-value used to define statistical significance was .05.

RESULTS

As indicated in Table 1, overall, 19 of 99 patients autografted after conditioning with IBu regimen (19%) had NPM1 mutation in the absence of FLT3 mutations. Three patients with NPM1+/FLT3− AML who failed to mobilize $CD34^+$ cells and were consolidated with 3 additional courses of high-dose ARA-C were excluded from the current analysis. The control group, accounting for 80 patients, included 16 cases (15%) with both mutations, 10 (12%) patients

Table 2. Toxicity and Therapeutic Results

	NPM1+	Controls	*P*-Value
Median number of months from CR to ASCT (range)	3 (2-5)	3 (2-6)	.87
Median number of $CD34^+$ cells infused ($\times 10^6$/kg)	6.5 (3.4-18.4)	6.7 (2.8-23.5)	.28
Median days to neutrophils recovery to $>0.5 \times 10^9$/L	11 (9-16)	12 (10-18)	.24
Median days to platelet recovery to $>20 \times 10^9$/L	18 (15-32)	19 (15-40)	.26
WHO nonhematologic toxicity episodes	16 (84%)	70 (87%)	.36
Treatment-related mortality	0	0	n.a.
Median DFS (months)	not reached	16	.007
Median OS (months)	not reached	25	.02
Relapse post-ASCT	3/19 (15%)	49/80 (61%)	.001

ASCT indicates autologous stem cell transplantation; CR, complete remission; DFS, disease-free survival; OS, overall survival.

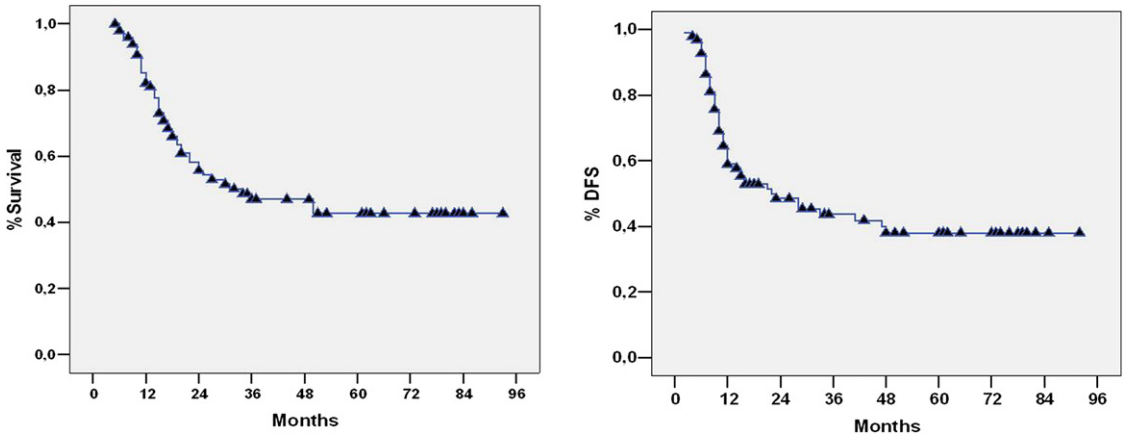

Figure 1. OS and DSF for the whole patient population (median: 34 and 22 months, respectively).

with only FLT3/ITD mutation, and 54 patients (68%) in whom neither NPM1 nor FLT3 mutations were detectable. Median WBC count (7.3 versus 14.8 $\times$ 10^9/L, $P = .003$), serum LDH (639 versus 875 IU/L, $P = .01$), percentages of PB (50% versus 75%, $P = .04$), and BM blasts (55% versus 80%, $P = .04$) were significantly higher in the NPM1+ patients compared to controls. Other clinical parameters, including median age and percentage of patients aged over 60 years, percentage of secondary AML, and FAB subtype, did not differ according to the presence or absence of NPM1 isolated mutation. However, AML post-MDS was diagnosed in only 1 patient with NPM1+ AML. Finally, percent of patients receiving intravenous Bu was similar in the 2 groups ($P = .35$).

Median time from CR achievement to ASCT was similar between the 2 groups (3 months, range: 2-5 for NPM1+ patients) as opposed to 3 for the control group (range: 2-6), $P = .87$), as shown in Table 2. In addition, time to neutrophil and platelet recovery to >0.5 $\times$ 10^9/L and 20 $\times$ 10^9/L after ASCT, respectively, was not different between the group of NPM1+ patients (11 versus 12 days, $P = .24$ for neutrophils; 18 versus 19 days for platelets, $P = .35$, respectively). Of note, no difference was recorded as nonhematological toxicity is concerned. More in detail, the incidence of oral mucositis, which was the most frequent adverse effect, occurred in 84% of NPM1 patients and 87% of controls, $P = .37$. The median number of $CD34^+$ cells infused were comparable between the 2 groups (6.5 $\times$ 10^6/kg for the NPM1+ subgroup, range: 3.4-18.4) as opposed to 6.7 (range: 2.8-23.5) for the control group, $P = .28$. No case of treatment-related mortality was recorded either in NPM1+ AML patients or in controls.

After a median follow-up for surviving patients of 36 months (range: 6-93), the median survival for the whole patient population was 34 months (95% confidence intervals [CIs] 12.1-55.8), whereas the median DFS was 22 months (95% CIs 8.2-38.2), as shown in Figure 1. As indicated in Figure 2, median OS and DFS were significantly longer for patients with isolated NPM1 mutation as opposed to controls (OS: not reached versus 25 months, $P = .02$; DFS: not reached versus 16 months, $P = .007$, respectively). Of interest, patients with isolated NPM1 mutation

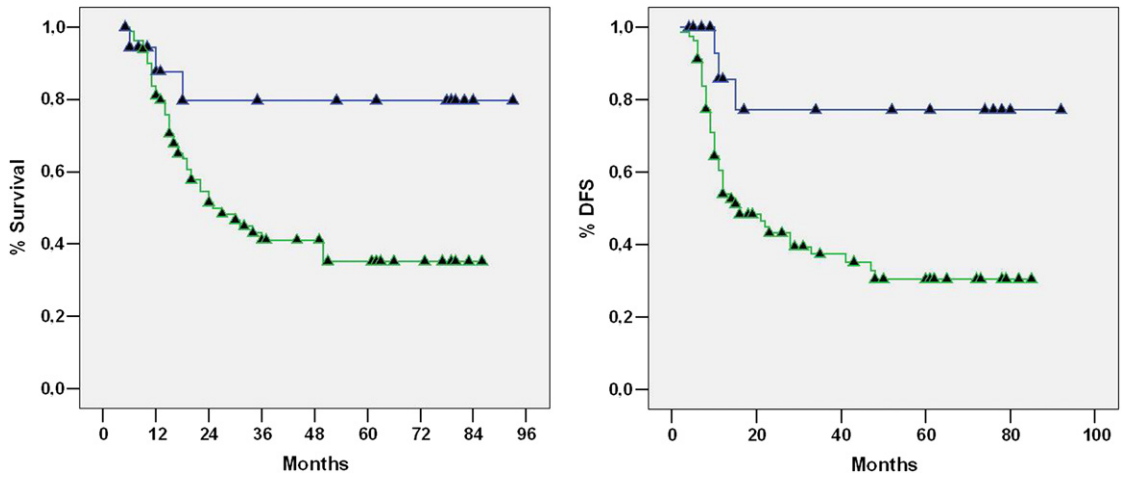

Figure 2. OS and DFS of patients with NPM1 isolated mutations as opposed to controls: median OS and DFS were significantly longer for patients with isolated NPM1 mutation as opposed to controls (OS: not reached versus 25 months, $P = .02$; DFS: not reached versus 16 months, $P = .007$, respectively).

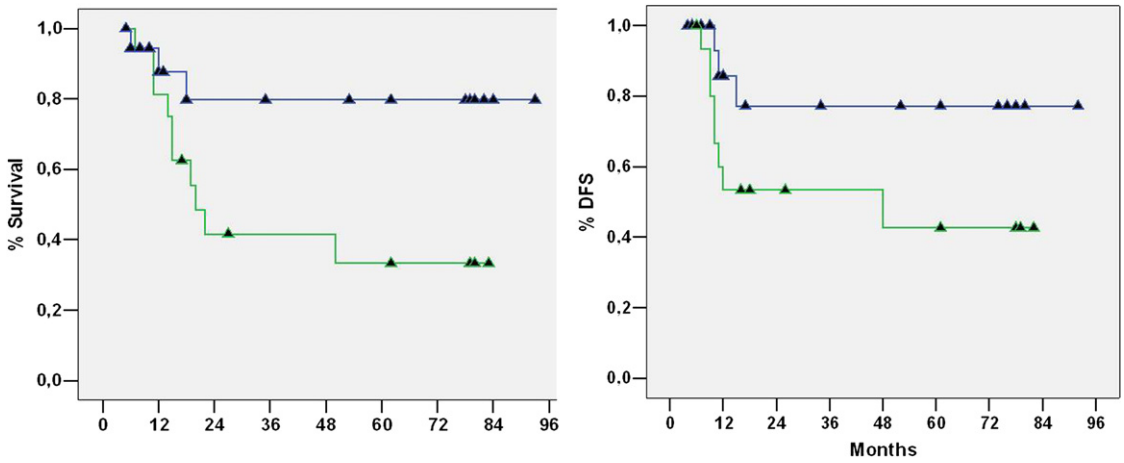

Figure 3. OS and DFS of patients with NPM1 isolated mutations as opposed to patients with NPM1+/FLT3+ AML (median OS not reached versus 27 months, $P = .03$; DFS not reached versus 16 months, $P = .004$).

had a better outcome in terms of either OS or DFS compared to the group of NMP1+/FLT3+ patients (median OS not reached versus 27 months, $P = .03$; DFS not reached versus 16 months, $P = .004$) as shown in Figure 3. Relapse post-ASCT occurred in 3 of 19 patients with NPM1 isolated mutation (16%), 2 with de novo AML and 1 with AML post-MDS, as opposed to 49 of 80 in the control group (61%), $P = .001$. Overall, second CR was achieved in 12 of 52 relapsed patients (23%) and was almost exclusively limited to those whose first CR lasted more than 6 months.

DISCUSSION

NPM1 gene mutations, which cause aberrant cytoplasmic expression of nucleophosmin, are the most frequent genetic alteration in AML, being found in about 30% cases [26]. NPM1 mutations are detected by molecular techniques or surrogates such as immunohistochemistry, Western blotting, and possibly fluorescent activated cell sorter analysis. These methods are complementary rather than competitive, and offer a flexible approach to diagnosis; therefore, investigation of NPM1 mutations is currently considered as essential for the routine diagnosis of AML [27]. Patients with NPM1-mutated AML usually respond well to induction CHT [28], with 75% to 80% achieving CR. Evidence of the high sensitivity of NPM1-mutated AML cells to chemotherapy derives from further clinical observations. In particular, NPM1, but not *FLT3-ITD* mutations predict early blast cell clearance and CR rate in patients with NK-AML or high-risk MDS [29], after conventional induction CHT. Moreover, increase sensitivity to chemotherapeutical agents has also been demonstrated by in vitro studies, as an effect of interaction between cytoplasmic NPM and NF-kB in the cytoplasm, resulting in the sequestration and inactivation of NF-kB [30].

In this study we retrospectively analyzed the prognostic relevance of isolated NPM1 mutation in a group of 99 patients autografted in first CR after conditioning with high-dose IDA and Bu. Such a regimen was specifically designed for AML, by replacing Cy, which is included in the classical Bu/Cy regimen with IDA and, in previous studies, provided very encouraging results in terms of reduction of relapse rate and CR duration, namely, in patients with intermediate cytogenetics [16-18]. In addition, BuI conditioning was found as able to overcome the adverse prognostic relevance of FLT3 mutations [31]. Overall, in our series 35 patients had NPM1 mutation (35%), 16 of whom are in association with FLT3/ITD mutation. As previously reported, most NMP1+ patients presented with de novo AML as well as with higher blast count either in BM or peripheral blood. On the contrary, no difference in age, sex, or FAB subtype were found. Therapeutic results are quite encouraging, given that after a median follow-up of 35 months from diagnosis, median OS and DFS have not been reached, and only 3 of 19 patients (16%) relapsed as opposed to over 50% in the control group. These favorable results suggest a peculiar efficacy of the BuI regimen in this AML patient subset, mainly of IDA, which was used at high dose as c.i. in the conditioning regimen. Even though no specific data have been published, either experimental or clinical evidence suggests particular sensitivity of NPM1+ AML blast cells to anthracyclines. In anaplastic large cell lymphoma, the fusion protein, nucleophosmin-anaplastic lymphoma kinase (NPM-ALK), results from the chromosome translocation t(2;5) (p23;q25) and is associated with favorable prognosis [32]. A recent study demonstrated that the downregulation of NPM-ALK resulted in decreased cell proliferation and increased cell apoptosis. When used in combination with chemotherapeutic agents, such as doxorubicin, the

inhibition of the NPM-ALK augments the chemosensitivity of the tumor cells [33]. In young adults with AML, intensifying induction therapy with a high daily dose of daunorubicin improved the rate of CR and the duration of OS compared with the standard dose [34]. Of note, clinical benefit was only observed in patients with intermediate cytogenetics, in whom most NPM1+ AML cases are found. Similar results were reported in older AML population, in which benefit of high-dose daunorubicin was limited to core binding factor AML as well as to AML with intermediate cytogenetics [35]. Taken together, these findings seem to suggest a peculiar sensitivity of NPM1+ myelogenous blasts to CHT, namely, to anthracyclines, and may account for the favorable results achieved in our NPM1+ AML patient series. In our study, the number of $CD34^+$ cells collected was relatively high (median 6.5 $\times$ 10^6/kg for NPM1+/FLT3− patients and 6.8 $\times$ 10^6/kg in the controls, respectively) and this in previous studies has been associated with poor prognosis in AML patients undergoing ASCT or not [36]. However, this value is lower than 10 $\times$ 10^6/kg, which was indicated as cutoff value for the prediction of unfavorable outcome [36]. In addition, it is conceivable that in NPM1+/FLT3− AML, patients with less leukemic cells could have contaminated the graft, given their peculiar sensitivity to induction and consolidation chemotherapy.

In conclusion, although the possible benefit of a conditioning regimen based on high-dose IDA does clearly need further investigation on a larger number of patients in a randomized trial, our results clearly suggest that patients with NPM1+/FLT3− AML are ideal candidates for high-dose consolidation with ASCT.

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